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New fungicide and bactericide Zeroxxe®: *in vitro* assessment of fungicidal and bactericidal activity

MARINA POBEDINSKAYA[1], SERGEY ELANSKY[1,5], LYUDMILA KOKAEVA[1], IRINA KUTUZOVA[1], INNA PRONICHEVA[1], MARIA KUZNETSOVA[2], BORIS KOZLOVSKY[2], ALEXANDER IGNATOV[3], PAVEL ZHEREBIN[1,4], ALBERT DENISOV[4], YURI KRUTYAKOV[1,4]

[1] Lomonosov Moscow State University, Leninskie gory, 1, Moscow, 119991, Russia
[2] All-Russian Research Institute of Phytopathology, Institute str., 5, Bolshie Vyazemy, Moscow region, 143050 Russia
[3] Centre «Bioengineering» of Russian Academy of Sciences, 60-Letiya Oktyabrya str., 7/1, Moscow, 117312, Russia
[4] Grand Harvest Research International Ltd., 7 Ronda Doctor Turró, 9-B Mataró, Barcelona, 08303, Spain
[5] All-Russian Lorkh Research Institute of Potato Farming, Lorkh str., Kraskovo-1, Moscow region, 140051 Russia 140051, Russia

Corresponding author: Sergey Elansky, e-mail: snelansky@gmail.com

SUMMARY

Zeroxxe® is a new fungicide and bactericide product with a broad spectrum of activity against phytopathogenic fungi, oomycetes and bacteria. In Russia, Zeroxxe® is in the final stage of the four-year registration process as the world's first fungicide and bactericide containing nanosilver as an active component. An active substance of Zeroxxe® is silver nanoparticles (silver NPs, 3000 ppm) stabilized with environmentally safe biodegradable amphoteric surfactant. In vitro studies of Zeroxxe® revealed its high efficacy against a wide range of fungal pathogens of potato: *Rhizoctonia solani* (black scurf), *Phytophthora infestans* (late blight), *Colletotrichum coccodes* (black dot), *Helminthosporium solani* (silver scurf), *Alternaria solani* and *A. alternata* (early blight), *Fusarium solani* (dry rot), *Sclerotinia sclerotiorum* (sclerotinia rot). To assess the bactericidal effect of Zeroxxe® six types of pathogenic bacteria were tested: *Pectobacterium carotovorum* (soft rot of potatoes and vegetables), *Dickeya dianthicola* (black leg and soft rot of potato), *Agrobacterium tumefaciens* (bacterial cancer of fruit, ornamental plants and grapes), *Xanthomonas vesicatoria* (bacterial black spot of tomato), *Clavibacter michiganensis* (bacterial cancer of tomato and potato), *Xanthomonas campestris* (vascular bacteriosis of cabbage and rape). 30 minutes incubation of bacteria in the solution of Zeroxxe® (100 ppm of silver NPs) completely inhibits growth of all bacteria. In the case of 10 ppm solution 45-85% of all colonies were inhibited. The results prove the potential of Zeroxxe® as a fungicide and bactericide for pre-plant treatment of various seeds and tubers, treatment of vegetating plants, as well as for the treatment of tuber and root crops before storage.

KEYWORDS
Fungicides, bactericides, antibiotics, potato diseases, silver nanoparticles.

INTRODUCTION

Modern technologies of agriculture imply intensive use of plant protection products at all stages of production. Nearly all chemical plant protection products are more or less toxic for mammals; they (or products of their further transformation) accumulate in organs of treated plants, in the soil, get into water and air, which leads to undesirable environmental consequences. That is why special attention is paid to the development of plant protection products harmless for mammals and plants.

Silver influences a wide range of biological processes in microorganisms. Silver nanoparticles (NPs) can be subject to slow oxidative dissolution in close proximity to the cell membrane of bacteria and fungi, generating silver ions and thus causing their death. *Escherichia coli* was used to demonstrate the effect of silver on proteins of ribosomal complex as well as proteins participating in glycolysis and the cycle of tricarboxylic acids, which damages the synthesis of ATP and leads to quick cell death (Yamanaka *et al.*, 2005). Wide spectrum of action of silver ensures its use against a big number of different pathogenic organisms with no fear of appearance of new resistant strains.

In the XIX-XX centuries silver-based plant protection products were not widespread because of their high price, unlike copper-based pesticides. Nowadays copper pesticides are among the most popular in the world, despite their high application rate, which sometimes reaches 10 kilograms per hectare, and the negative impact on the environment. However, the advances in the synthesis and stabilization of silver NPs resulted in appearance of plant protection products with high efficiency at very low concentrations of active components, which makes their use economically justified and minimizes environmental risks related to their use.

Fungicidal effect of silver NPs was demonstrated in a number of researches. American researchers (Jo *et al.*, 2009) proved their high efficiency against germinating spores of *Bipolaris sorokiniana* (Sacc.) Shoem and *Magnaporthe grisea* (Hebert) Barr. The same research shows good prospects of application of NPs as a contact fungicide for protection of leaves of vegetating plants of ryegrass. Korean authors (Lamsal *et al.*, 2011) showed the effect of silver NPs on different agents of anthracnose (*Colletotrichum* sp.). The comparison of field and laboratory experiments showed that NPs are able to inhibit the development of *Colletotrichum* sp. in pepper plants in fields with the same efficiency as *in vitro*. In other research papers (Kim *et al.*, 2012, Kim *et al.*, 2009, Min *et al.*, 2009) high fungicidal effect of NPs against more than 20 phytopathogenic fungi was demonstrated, while the testing was conducted in different nutrient media.

The experience of different scientific groups showed that compositions based on colloidal silver stabilized with compounds of various chemical classes were efficient in most cases against phytopathogenic fungi and bacteria. That is why in 2012 the researchers of the Lomonosov Moscow State University and Grand Harvest Research International Ltd. have begun the development of the world's first fungicide and bactericide containing nanosilver as a main active component. This pesticide under trade name Zeroxxe® was tested in Russia to analyse its fungicidal and bactericidal activity in laboratory experiments and in field trials on many crops, its acute and chronic toxicity for animals, environmental impact, and many other parameters.

This article contains the results of the laboratory assessment of the fungicidal and bactericidal activity of Zeroxxe® against most serious fungal and bacterial phytopathogens.

MATERIALS AND METHODS

Samples of fungi *Colletotrichum coccodes* (Wallr.) S. Hughes, *Helminthosporium solani* Durieu & Mont., *Rhizoctonia solani* J.G.Kühn, *Fusarium solani* (Mart.) Sacc., *Alternaria alternata* (Fr.) Keissl. and *A. solani* Sorauer, oomycete *P. infestans* (Mont.) de Bary were isolated from infected potato tissue. Isolate of *Sclerotinia sclerotiorum* (Lib.) de Bary was isolated from the affected carrot. For the experiment one isolate of each species of fungi was taken.

Laboratory evaluation of fungicidal properties of Zeroxxe®

The resistance of isolates to fungicides was assessed on pea agar medium, supplemented with the corresponding fungicide at various concentrations (0.1, 1, 10, 100 ppm (=μg/ml) of silver NPs), and on the medium without fungicide (control). An agar piece containing fungi (5 mm in diameter) was placed into the centre of the Petri dish, which was then sealed with a paraffin film (Parafilm). Petri dishes were incubated at temperature of 23-25°℃ under natural light. The diameter of colonies was measured when the diameter of the fungi colony in the fungicide-free control sample achieved 0.7-0.75 of the diameter of the Petri dish. The radial growth on each concentration of fungicide was assessed in three replications (each isolate was put into 3 Petri dishes with the same fungicide concentration). On the basis of the averaged values of diameters of colonies the ratio of the sizes of colonies in the medium with fungicide and in the medium without fungicide were calculated. For each isolate the value of the effective concentration EC_{50} was determined, i.e. the concentration of fungicide required to slow down the speed of the radial growth of the colony twice with 50%.

Influence of Zeroxxe® on germination of zoosporangia P. infestans with zoospores (indirect germination)

Silver NPs stabilized with amphoteric surfactant (an active substance of Zeroxxe®) in concentration of 25 and 100 ppm were used in microbiological experiments in the form of aqueous dispersions. Fluazinam in concentration of 500 ppm was used as etalon fungicide, which corresponds to its recommended concentration in tank mixtures for treatment of vegetating plants. In our experiment germination of zoosporangia of four phytopathogenic isolates of *P. infestans* obtained from affected leaves of potato was analysed. The suspension of zoosporangia of *P. infestans* was obtained by means of washout from 8-days old pathogenic culture with distilled water. Then the suspension was mixed with the same amount of silver NPs or fluazinam solution in order to obtain aforementioned concentrations of active substances in culture media. The germinated (empty) zoosporangia were counted after 3 hours of cultivation at +10°C. In each sample 600 zoosporangia (6 samples, 100 pcs. on each) were accounted. The percentage of germinated zoosporangia was determined.

Estimation of bactericidal effect

For the estimation of bactericidal effect the bacteria were grown in agar nutrient medium during 24 hours at +28°℃ and then washed with sterile distilled water. Suspensions with concentration of 1000 CFU/ml were used for microbiological experiments. Bacterial suspension was mixed with Zeroxxe®-containing water solution (final concentration 10 and 100 ppm of silver NPs) and incubated for 30 min. Control samples were incubated with the similar volume of distilled water. After the incubation 50 μl of suspension were resuspended over the surface of agar nutrient medium. Colonies were counted after 48 and 72 h of incubation under +28°℃ and compared to the fungicide-free control sample.

RESULTS AND DISCUSSION

Laboratory evaluation of fungicidal properties of Zeroxxe®

The results show suppression of the radial growth of colonies of all tested species of fungi after addition of Zeroxxe® in the concentration of silver NPs more than 10 ppm (Table 1). High efficiency against *Rhizoctonia solani*, *Phytophthora infestans*, *Colletotrichum coccodes*, *Helminthosporium solani, Alternaria solani* and *Sclerotinia sclerotiorum* was noted. Fungicidal effect against *Alternaria alternata* and *Fusarium solani* was weaker. Differences of the efficiency of fungicides against strains *A. solani* and *A. alternata* correspond to our previous research: well known fungicides mancozeb, azoxystrobin, chlorothalonil were also less effective against *A. alternata* than against *A. solani* (Pobedinskaya *et al.*, 2012).

Table 1. *Influence of Zeroxxe® on radial growth of colonies of tested fungi*

Fungi/ oomycetes	Ratio of diameters of the colony on the media with different concentration of silver NPs and the fungicide-free control (in %)					EC_{50}*, mg/l
	0 (control)	0.1 ppm	1 ppm	10 ppm	100 ppm	
Phytophthora infestans	100	90**	55	33	0	3.1
Rhizoctonia solani	100	95	78	2	0	0.4
Fusarium solani	100	95	91	41	33	8.3
Colletotrichum coccodes	100	96	94	23	0	6.6
Helminthosporium solani	100	97	83	50	10	10
Alternaria alternata	100	93	107	52	41	28
Alternaria solani	100	92	92	35	22	7.7
Sclerotinia sclerotiorum	100	93	73	0	0	3.9

* - the concentration of a fungicide, causing a 50% delay in the colony growth rate as compared to fungicide-free control

** - the diameter of colonies was measured when the diameter of the fungi colony in the fungicide-free control sample achieved 0.7 to 0.75 diameter of a Petri dish.

Influence of Zeroxxe® on indirect germination of zoosporangia of P. infestans

According to the experimental results, Zeroxxe® certainly decreased germination of zoosporangia. In the control sample the average number of germinated (empty) zoosporangia in the visual field of the microscope at 150-fold magnification comprised 54-80 pcs, in the samples inoculated with 25 ppm of silver NPs – 0.2-12; at 100 ppm – 0.3-2.3 (Table 2). The maximum average number of germinated zoosporangia in the experiment with the etalon fungicide fluazinam reached 0.3. Thus, Zeroxxe® reduced germination of zoosporangia *P. infestans* at the level of fluazinam, though the concentrations of silver were lower.

Table 2. *Influence of Zeroxxe® on indirect germination of zoosporangia of P. infestans*

Variant	Average number of germinated zoosporangia in the visual field of the microscope at 150-fold magnification				
	Strain 1	Strain 2	Strain 3	Strain 4	Medium
Control (water)	69	80	60	54	65.8
Silver NPs 25 ppm	0.5	0.7	12	0.2	3.4
Silver NPs 100 ppm	0.5	0.3	2.3	0.3	1.4
Fluazinam 500 ppm	0.3	0	0	0	0.1
Least significant difference 0.95	5.3	5.0	5.4	5.2	5.2

Influence of Zeroxxe® on the growth of phytopathogenic bacteria

Incubation of bacteria in the solution of Zeroxxe® with silver NPs concentration of 100 ppm for 30 min completely inhibited the growth of colonies of the bacteria, while 10 ppm of silver NPs reduced the number of colonies by 45-85%. The number of colonies of *Dickeya dianthicola* and *Agrobacterium tumefaciens* significantly decreased even after the incubation with Zeroxxe® at silver NPs concentration of 1 ppm (Table 3).

Table 3. *Inhibition of growth of phytopathogenic bacteria after the incubation in Zeroxxe® solution for 30 min*

Species of bacterium	The number of colonies on the agar media after the incubation in Zeroxxe® solution with different concentrations of silver NPs, % of control		
	1 ppm	10 ppm	100 ppm
Pectobacterium carotovorum	87	45	0
Dickeya dianthicola	61	23	0
Agrobacterium tumefaciens	75	15	0
Xanthomonas vesicatoria	90	38	0
Xanthomonas campestris	97	55	0
Clavibacter michiganensis	92	38	0

Our experiments revealed high fungicidal and bactericidal activity of Zeroxxe®. The results of assessment of fungicidal effect of Zeroxxe® coincided in a number of parameters with the data obtained during assessment of fungicidal activity of silver NPs in other laboratories. Thus, in our research EC_{50} of silver NPs for the majority of the tested fungi was within the range 3.1-10 ppm; it reached its maximum for *A. alternata* and comprised 28 ppm. In the researches of other authors EC_{50} for non-stabilized silver NPs was as follows: for *Bipolaris* sp. 4.8-8.8 ppm, for *Magnaporthe grisea* 3.9-4.7 ppm, *A. alternata* (EC_{50}=38 ppm), *A. solani* (less than 10 ppm), *Fusarium* (9-55 ppm for different species), *Pithium* sp. (about 2 ppm), *Colletotrichum* (8-100 ppm for different species) (Jo *et al.*, 2009, Kim *et al.*, 2012, Lamsal *et al.*, 2011). For germinating sclerotia nanoparticles were more toxic than for mycelium: S*clerotinia sclerotiorum* (EC_{50}=1 ppm) and *Rhizoctonia solani* (less than 1 ppm). EC_{50} for silver NPs against

S. *sclerotiorum* (7 ppm) and *R. solani* (6 ppm) (Min *et al.*, 2009) exceeded EC_{50} revealed in our research (3.9 and 0.4 ppm respectively).

In general, the results on EC_{50} presented in the cited research papers are close to the ones determined for Zeroxxe® in our laboratory, but in most cases were higher. Evidently, the surface modification of nanoparticles increases the fungicidal effect.

It should be noted that as Zeroxxe® is almost completely harmless for animals and plants and not dangerous to the environment in recommended doses, it can be recommended for application in areas where the use of toxic pesticides is not permissible. For instance, it can be applied for treatment of potato (including ware and technical potato) tubers before placing for storage, during storage, and before planting, treatment of plants in glass houses and private kitchen-gardens.

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